ORIGINAL ARTICLE 10.1111/j.1469-0691.2005.01262.x

Efficacy of high loading doses of liposomal amphotericin B in the treatment of experimental invasive pulmonary aspergillosis

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ABSTRACT

This study aimed to investigate whether initial treatment of experimental pulmonary aspergillosis with high loading doses can be used as an alternative to standard therapeutic regimens. Steroid-immunosuppressed rats, infected intratracheally with *Aspergillus fumigatus*, received either amphotericin B deoxycholate (d-AmB) 1 mg/kg/day, liposomal amphotericin B (L-AmB) 5 mg/kg/day, or underwent a 3-day course of L-AmB 10 mg/kg, or 10 mg/kg for the first 3 or 4 days of treatment, followed by 3 mg/kg until the end of treatment. Therapy started 24 h after fungal challenge and lasted for 7 days. Compared to controls, survival was improved significantly in animals receiving any L-AmB regimen ($p \leq 0.003$), but not d-AmB. Compared with d-AmB, L-AmB at initial doses of 10 mg/kg followed by 3 mg/kg/day was consistently more effective, but only when measured in terms of survival, lung weight and glucosamine levels, and not log CFU. Despite the absence of significant differences between any of the L-AmB regimens, a trend towards better response rates with the higher loading dose was observed.

Keywords Amphotericin B, animal model, aspergillosis, invasive disease, loading doses, therapy

Original Submission: 19 November 2004; **Revised Submission:** 22 April 2005; **Accepted:** 31 May 2005

Clin Microbiol Infect 2005; 11: 999–1004

INTRODUCTION

Liposomal amphotericin B (L-AmB) has become a therapeutic alternative to amphotericin B deoxycholate (d-AmB) for the treatment of invasive pulmonary aspergillosis (IPA), which is an infection that is generally lethal when left untreated [1]. Current recommendations for the treatment of systemic aspergillosis include the use of d-AmB 1–1.5 mg/kg/day, L-AmB 5 mg/kg/day, or voriconazole, as first-line therapy, with caspofungin as an alternative [2–4]. However, there is still a need for new strategies in the treatment of invasive aspergillosis in order to diminish the current high rates of therapeutic failure. The maximum dose of d-AmB recommended for administration to humans is 1.5 mg/kg/day because of intolerance and other adverse effects [5]; however, L-AmB has lower toxicity than d-AmB, thus allowing the infusion of significantly higher doses. Animal models of invasive aspergillosis have shown outcome benefits (i.e., increased survival, reduction of fungal load) with the use of L-AmB 10 mg/kg/day [6–8]. A recent study conducted in humans found that doses up to 15 mg/kg were safe and well-tolerated [9], and preliminary results from a pilot prospective open-label study have shown that L-AmB 10 mg/kg/day may be a safe and effective alternative for the management of invasive aspergillosis in immunocompromised patients (43rd Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, 2003, abstract M-966).

In addition to reduced toxicity, the incorporation of amphotericin B into lipid vesicles [10] has modified the pharmacokinetics of the drug with respect to the conventional deoxycholate-associated formulation of amphotericin B [11], although the impact of these changes on the treatment of lung infection by *Aspergillus fumigatus* is still

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unknown. Moreover, it has been suggested that the pharmacokinetic profile means that an improved treatment outcome might be achieved with administration of high loading doses [12]. The present experimental study investigated whether initiating treatment for IPA with L-AmB 10 mg/kg, a level associated with maximum plasma concentrations in humans [9], and then continuing with lower doses, may represent an alternative to the classical treatment with d-AmB 1 mg/kg/day. In addition, the efficacy of initial high doses followed by low doses was compared with that of L-AmB 5 mg/kg/day in a steroid-immunosuppressed rat model of infection.

MATERIALS AND METHODS

Strain

A clinical isolate of *A. fumigatus*, strain AFJJ (deposited at the National Centre for Microbiology, Instituto de Salud Carlos III, Madrid, Spain) was used throughout the study. The strain was maintained on Sabouraud dextrose agar (SDA) slants at – 80°C. The susceptibility of this strain to amphotericin B was 1 mg/L when determined according to NCCLS guidelines [13].

Animals

Female SPF Wistar rats weighing 180–200 g (Harland Iberica, Barcelona, Spain) were housed in cages with HEPA filters and fed with a protein 8% w/w diet and sterile water *ad libitum*. The experimental protocol was approved by the Ethics Committee of Vall d'Hebron Hospitals and the Autonomous Government of Catalonia.

Animal model

The study was carried out in an animal model of IPA, described previously [14]. Briefly, animals were immunosuppressed with subcutaneous cortisone acetate 125 mg/kg (Sigma, St Louis, MO, USA) three times/week, starting 14 days before the fungal challenge and continuing to the end of the experiment. To avoid bacterial superinfection during immunosuppression, rats were given colistin 2500 IU/mL in drinking water, and cefepime 150 mg/kg twice-daily subcutaneously. Four days before the infection, a sterile silastic catheter connected to a subcutaneous port was inserted surgically through the right jugular vein into the inferior cava vein for the administration of antifungal treatment [15]. All surgical procedures were performed under general anaesthesia (ketamine 100 mg/kg plus xylazine 10 mg/kg, intramuscularly).

After six doses of steroids (day 0), rats were infected via a tracheotomy with 0.3 mL of a freshly prepared conidial suspension harvested from a subculture of the *A. fumigatus* strain grown on SDA plates. Conidia were harvested with a sterile pipette by flooding the plates with sterile saline containing Tween-20 0.025% v/v. The resulting suspension was washed in sterile phosphate-buffered saline, counted in a haemocytometer, and adjusted in sterile saline to a final concentration of 8×10^6 conidia/mL. Serial ten-fold dilutions were plated on to SDA plates for assessment of purity, size and viability of the inoculum. At least 95% of conidia in the inoculum were viable as assessed by colony counts.

Antifungal therapy

Eight doses of antifungal treatment were administered intravenously, starting 24 h after infection. Animals were assigned randomly to receive either dextrose 5% w/v (control, $n = 52$), d-AmB (Fungizone; Bristol-Myers Squibb Group, Barcelona, Spain) 1 mg/kg/day ($n = 28$), L-AmB (AmBisome, Gilead Sciences, Madrid, Spain) 5 mg/kg/day ($n = 33$), L-AmB 10 mg/kg/day for 3 days ($n = 34$), or L-AmB 10 mg/kg/day for 3 days ($n = 34$) or 4 days ($n = 21$), followed by L-AmB 3 mg/kg/day until the end of treatment. Antifungal drugs were prepared immediately before use, according to the manufacturers' instructions.

Assessment of antifungal efficacy

Animals were inspected every 4 h from 08.00 h to 00.00 h, and mortality was recorded throughout the experiment. Surviving rats were killed humanely 24 h after the last dose of antifungal treatment. Animals were dissected immediately after death. Lungs were removed aseptically, weighed and homogenised in 10 mL of sterile distilled water for 15 s with a tissue homogeniser (Ultraturrax T25 basic; IKA Labortechnik, Staufen, Germany). Aliquots (500 μL) of homogenates were diluted serially and plated on to SDA and trypticase soy blood agar plates to assess fungal growth quantitatively and to rule out bacterial superinfection. Data from animals dying during the night could introduce a confounding factor in post-mortem multiplication, although this factor was equal in all groups.

Lung homogenates were processed for chitin assays to quantify the fungal burden in lungs [16]. With this technique, chitin is depolymerised, deaminated and deacetylated to glucosamine monomers [17]. In brief, homogenates were centrifuged, resuspended in 4 mL of 0.1 M sodium lauryl sulphate (Sigma), heated at 100°C for 15 min and left to cool. Pellets were washed in distilled water, resuspended in 3 mL of 21.39 M KOH (Sigma) and heated at 130°C for 1 h. After addition of 8 mL of ice-cold ethanol 75% v/v, tubes were kept in ice-cold water for 15 min, and then 0.3 mL of a suspension of diatomaceous earth (Celite 545, Sigma) in ethanol 75% v/v was added. Pellets were washed once with ice-cold ethanol 40% v/v, and twice with distilled water. The supernatants were discarded, the resultant pellets were resuspended in distilled water to a volume of 0.5 mL, and 0.5 mL of 0.72 M $NaNO_2$ and 0.5 mL of 0.37 M $KHSO_4$ were then added. The tubes were centrifuged and volumes of the supernatants were mixed with 0.2 mL of 1.1 M ammonium sulphamate, followed by 0.2 mL of 3-methylbenzothiazolone hydrazone hydrochloride monohydrate (Sigma), and heated for 3 min. The supernatants were left to cool, 0.2 mL of 0.03 M $FeCl_3.6H_2O$ was added, and the optical density was read in a spectrophotometer (UV-160A; Shimadzu Corp., Kyoto, Japan) at 650 nm after 25 min.

Statistical analysis

Survival was analysed with Kaplan–Meier plots and the log-rank test. Lung weight (g), pulmonary chitin content ($\log_{10}$ μg glucosamine/paired lungs) and $\log_{10}$ CFU were compared

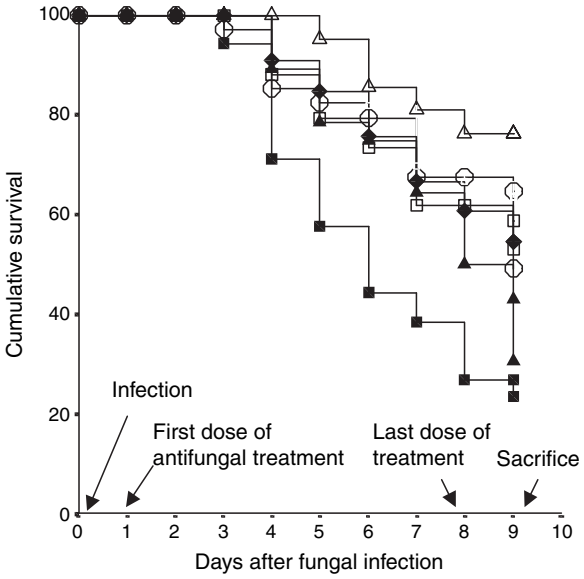

Fig. 1. Effect of amphotericin B deoxycholate (d-AmB) vs. different regimens of liposomal amphotericin B (L-AmB) or control on the cumulative survival of steroid-immunosuppressed rats with invasive pulmonary aspergillosis. Filled squares, control; filled triangles, d-AmB 1 mg/kg/day; filled diamonds, L-AmB 5 mg/kg/day; open squares, L-AmB 10 mg/kg/day for 3 days; open circles, L-AmB 10 mg/kg/day for 3 days, followed by L-AmB 3 mg/kg/day; open triangles, L-AmB 10 mg/kg/day for 4 days, followed by L-AmB 3 mg/kg/day.

using the Mann–Whitney *U*-test. Unless indicated otherwise, results were expressed as the mean and 95% CI of the mean, with $p \leq 0.05$ considered significant.

No correction for multiple comparisons was used.

RESULTS

Data from the four experiments performed were pooled, as no statistically significant differences were found between the controls of each experiment.

Survival of animals receiving antifungal treatment was improved compared with the control group, with significant differences for groups receiving L-AmB treatment: $p < 0.003$ for L-AmB 5 mg/kg/day and L-AmB 10 mg/kg/day for 3 days (Fig. 1); $p < 0.002$ and $p < 0.001$, respectively, for L-AmB 10 mg/kg/day for 3 or 4 days, followed by 3 mg/kg/day until the end of treatment. No differences were found between the L-AmB-based regimens; however, L-AmB 10 mg/kg/day for 4 days followed by 3 mg/kg/day was the only regimen that resulted in significantly increased survival with respect to the d-AmB regimen ($p < 0.009$).

Results observed in animals that survived ≥5 days of antifungal treatment are summarised in Table 1. There was a significant reduction in lung weight, which is a marker of fungus-related tissue injury, in all the groups receiving L-AmB when compared with the controls (p 0.001 for L-AmB 5 mg/kg/day; $p < 0.001$ for L-AmB 10 mg/kg/day for 3 days, and for L-AmB 10 mg/kg/day for 3 or 4 days, followed by 3 mg/kg/day). Again, although there were no differences between the groups receiving L-AmB 5 mg/kg/day throughout and those receiving high loading doses, the lung weights of the animals in the groups given L-AmB 10 mg/kg/day for 3 or 4 days, followed by 3 mg/kg until the end of the experiment, were significantly lower than those of animals receiving d-AmB (p 0.016 and p 0.009, respectively). With respect to fungal load, the chitin content in the lungs was found to be lower in rats receiving antifungal treatment than in controls. Statistically

Table 1. Results obtained with the different treatment groups

			Animals surviving treatment for ≥5 days			
Group	**Total**	**Survival: mean (95% CI)**	***n* (%)**	**Weight of paired lungs (g): mean (95% CI)**	**Log_{10} μg glucosamine/ paired lungs: mean (95% CI)**	**Log_{10} CFU/g of lungs: mean (95% CI)**
Control	52	5.60 (4.90–6.29)	23 (44.23)	2.87 (2.42–3.31)	2.11 (1.87–2.35)	5.40 (5.17–5.62)
d-AmB 1 mg/kg/day	28	7.00 (6.14–7.86)	21 (75)	2.30 (1.91–2.69)	2.06 (1.82–2.30)	5.19 (4.93–5.44)
L-AmB 5 mg/kg/day	33	7.33 (6.56–8.10)[a]	25 (75.76)	1.87 (1.56–2.19)[a]	1.88 (1.61–2.15)	5.22 (5.01–5.44)
L-AmB 10 mg/kg/day for 3 days	34	7.24 (6.42–8.05)[a]	25 (73.53)	1.84 (1.57–2.10)[a]	1.97 (1.69–2.24)	5.22 (4.94–5.51)
L-AmB 10 mg/kg/day for 3 days + 3 mg/kg/day[b]	34	7.44 (6.61–8.27)[a]	27 (79.41)	1.76 (1.43–2.10)[a,c]	1.74 (1.49–1.99)[a]	5.18 (4.99–5.37)
L-AmB 10 mg/kg/ day for 4 days + 3 mg/kg/day[d]	21	8.14 (7.39–8.89)[a,c]	18 (85.71)	1.61 (1.31–1.91[a,c]	1.59 (1.29–1.90)[a,c]	5.02 (4.74–5.31)[a]

L-AmB, liposomal aphotericin B; d-AmB, amphotericin B deoxycholate.
[a]$p \leq 0.05$ with respect to controls.
[b]L-AmB 10 mg/kg/day for 3 days, followed by 3 mg/kg/day until the end of therapy.
[c]$p \leq 0.05$ with respect to d-AmB 1 mg/kg/day.
[d]L-AmB 10 mg/kg/day for 4 days, followed by 3 mg/kg/day until the end of therapy.

significant differences with respect to the controls were evident only in groups that received doses of L-AmB 10 mg/kg/day for the first 3 (p 0.020) or 4 (p 0.009) days, followed by 3 mg/kg/day, but only the latter was significantly different from the d-AmB group (p 0.028). Outcome in the group in which L-AmB was suspended after 3 days tended to be poorer than in the animals that continued to receive low doses of L-AmB to the end of treatment, but no significant differences were found among these groups. As summarised in Table 1, the CFU/g of lung count was similar in nearly all the groups. Nevertheless, colony counts were significantly lower in animals that had received L-AmB 10 mg/kg/day for the first 4 days, plus 3 mg/kg/day until the end of treatment, than in controls (p 0.048).

DISCUSSION

The aim of this study was to determine whether initiating treatment for IPA with high doses of L-AmB and continuing with lower doses is a valid alternative to conventional regimens, i.e., administration of d-AmB 1 mg/kg/day or L-AmB 5 mg/kg/day. The initial dose of L-AmB was 10 mg/kg/day, which has been shown to yield the maximum plasma C_{max}, AUC_{24} and AUC_{∞} values in humans [9]. Experimental models of IPA using neutropenic and steroid-immunosuppressed rats have shown that this dosage is effective in increasing survival, reducing fungal load and pulmonary damage [6–8], and preventing fungal dissemination [7]. Administration of this dose was limited to a maximum of 4 days, based on a previous observation that use of this dose for ≥5 days in uninfected steroid-treated animals produces a drug interaction causing toxicity of unknown origin that contributes to the mortality, whereas lower doses do not have this effect [8]. Kisch *et al.* [18] reported in 1978 that co-administration of d-AmB and cortisone acetate produced renal lesions that were dose-related in severity, but there appears to be no similar information relating to L-AmB in the scientific literature.

The indications of the therapeutic efficacy of high loading doses of L-AmB were two-fold. First, in the steroid-immunosuppressed murine model of IPA, the use of d-AmB 1 mg/kg/day did not improve the outcome of the infection compared with the controls, whereas any of the studied regimens involving administration of L-AmB achieved statistically significant differences in at least one of the four parameters examined compared with the controls. This is consistent with previous work, in which the efficacy of ≥5 mg/kg/day of L-AmB has been observed [8]. Equivalent improved efficacy of ≥5 mg/kg/day of L-AmB compared with the conventional formulation has been reported in animal models of invasive aspergillosis [6,7], although in humans, the superiority of relatively high doses of L-AmB (i.e., up to 4 mg/kg/day) over d-AmB (up to 1.5 mg/kg/day) is still a topic of debate [19] because of difficulties in design, development and comparison of clinical trials. Interpretation of results involving the use of L-AmB are complicated further by the reported substantial inter-patient differences in the handling of this drug [20].

Second, despite the absence of differences in outcome between the various L-AmB regimens used in this study, a slight trend towards better results was observed in groups receiving a combination of loading doses followed by low doses of L-AmB, with prolonged administration of high loading doses followed by lower doses resulting in significant differences in survival, reduction of lung weight and glucosamine content compared with d-AmB 1 mg/kg/day. This was in contrast with the results obtained with L-AmB 5 mg/kg/day, but is in keeping with the findings of Becker *et al.* [21], who showed an improvement in survival and pulmonary fungal load in a neutropenic rat model of IPA with the use of another type of intensive initial therapy involving the addition of d-AmB on the first day of therapy with high doses of L-AmB.

In contrast to the results for survival, lung weight and lung glucosamine content, CFU counts did not vary significantly between the treatment groups, with the exception indicated in Table 1. This may be because CFU counts do not represent the real viable fungal load, as larger hyphae represent a larger fungal load, but not necessarily a larger number of CFUs [6,21].

The use of initial high doses for treating IPA represents an aggressive approach to therapy for this infection, the importance of which has been noted previously [21–23]. Although no substantive differences were found between the outcomes with any of the L-AmB regimens in the present experimental model of IPA, a trend towards a slightly better outcome was seen with the higher loading dose. The clinical meaning of this

observation remains to be determined, and caution in interpreting this result is required until further studies are conducted. However, preliminary results from a non-comparative study have shown that a primary treatment period consisting of 5 days of L-AmB 10 mg/kg/day, followed by 9 days of L-AmB 3 mg/kg/day, yields an overall response rate (both partial and complete) of 60% in patients with proven or probable invasive fungal infection or chronic disseminated candidiasis (Annual Scientific Meeting of the British Society for Haematology, Cardiff, 2004, abstract 202). The present study was not designed to determine the exact duration of initial therapy with high doses, and the suggested approach to treatment of IPA with loading doses should be the subject of in-depth investigations in other models of experimental IPA.

In conclusion, the use of initial high loading doses appears to be a valid alternative to d-AmB 1 mg/kg/day in the steroid-immunosuppressed model of IPA, with an efficacy that is at least equivalent to that of L-AmB 5 mg/kg/day. The duration of the initial high doses cannot be deduced from the findings in this study, and further work is required on this subject.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Fondo de Investigaciones Sanitarias (FIS exp. no. 01/1412) and was presented, in part, at the 42nd Interscience Conference on Antimicrobial Agents and Chemotherapy (San Diego, CA, USA, 2002). We thank C. Cavallo for English language assistance.

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